MCN

Molecular and Cellular Neuroscience **11,** 64–76 (1998)
Article No. CN980666

Cloning and Expression of a Novel Neurotrophin, NT-7, from Carp

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Neurotrophins have been demonstrated to play important roles in the development and functioning of the nervous system. This family of proteins consists of four homologous members in mammals: NGF, BDNF, NT-3, and NT-4/5. A new member, called NT-6, was recently cloned from the platyfish *Xiphophorus maculatus.* This protein shares closer structural relationship to NGF than the other neurotrophins, but contains a characteristic insertion of 22 amino acids that constituted the heparin-binding domain. Here we report the cloning of a novel neurotrophin from the fish *Cyprinus carpio* (carp), which shared about 66% amino acid identity to *Xiphophorus* NGF and NT-6. The neurotrophin, designated NT-7, possesses structural characteristics common to all known neurotrophins, such as the presence of six conserved cysteine residues and the flanking conserved sequences. In addition, there is an insertion of 15 amino acids at the position corresponding to that observed for NT-6. The neurotrophic activity of NT-7 was demonstrated by its ability to promote neurite outgrowth and neuronal survival of chick dorsal root ganglia. Phosphorylation assay of various Trk receptors overexpressed in fibroblasts suggested that NT-7 could activate TrkA but not TrkB or TrkC. Northern blot analysis revealed that NT-7 was predominantly expressed in peripheral tissues, though weak expression was also detected in the brain. Like NT-6, this novel neurotrophin might represent yet another NGF-like neurotrophin in lower vertebrates.

Key Words: **neurotrophin-7; nerve growth factor; neurotrophin-6; Trk receptors; neurite outgrowth; neuronal survival.**

INTRODUCTION

Neurotrophin is a family of proteins which plays important roles in the development and maintenance of the nervous system (for review, see Davies, 1994). This family consists of four members in mammals that share high homology to each other (approximately 50% amino acid identity). The prototypic nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) were isolated from snake venom and pig brain, respectively (Cohen *et al.*, 1954; Barde *et al.*, 1982). Subsequent cloning of the BDNF gene (Leibrock *et al.*, 1989) revealed its structural similarity to NGF, leading to the concept of the neurotrophin family. Using a homology cloning approach rather than protein purification, two additional members, neurotrophin-3 (NT-3) (Ernfors *et al.*, 1990; Hohn *et al.*, 1990; Jones and Reichardt, 1990; Maisonpierre *et al.*, 1990; Rosenthal *et al.*, 1990; Kaisho *et al.*, 1990) and neurotrophin-4/5 (NT-4/5) (Hallböök *et al.*, 1991; Berkemeier *et al.*, 1991; Ip *et al.*, 1992) were subsequently identified. This family of proteins was found in a wide range of vertebrates, from cartilaginous fish to mammals (Hallböök *et al.*, 1991).

The neurotrophins bind to two classes of receptors: the p75 receptor binds to neurotrophin with low affinity (for review, see Chao, 1994), and higher affinity of binding is observed with a family of receptor tyrosine kinases called Trks (for review, see Ip and Yancopoulos, 1996). While the p75 receptor binds to NGF, BDNF, and NT-3 with similar affinity (Rodríguez-Tébar *et al.*, 1992), different neurotrophins activate different Trk receptors: NGF specifically activates TrkA (Kaplan *et al.*, 1991); BDNF and NT-4/5 interact with TrkB (Klein *et al.*, 1991, 1992; Ip *et al.*, 1992), whereas NT-3 preferentially activates TrkC (Lambelle *et al.*, 1991; Soppet *et al.*, 1991; Squinto *et al.*, 1991; Ip *et al.*, 1993). Because of the Trk receptor specificities, different neurotrophins act on distinct neuronal populations, as revealed by both *in vitro* and *in vivo* studies (for reviews, see Korsching, 1993; Snider, 1994).

All the neurotrophins are basic proteins of about 120 amino acids being processed from larger precursors. There are some structural features common to all members of the neurotrophin family. These include encoding of the whole precursor protein in a single exon, the basic

1044-7431/98 $25.00

amino acids at the end of the pre–pro region that are important in proteolytic cleavage during processing of the precursor molecule, and the presence of six cysteine residues that are thought to maintain three-dimensional conformation of the molecule by disulfide bond formation. Moreover, there are conserved regions, mainly around the cysteine residues, that contain similar amino acid sequences in all neurotrophins. Taken together, these structural features define useful criteria in the identification of novel neurotrophins.

Recently, a new member of the neurotrophin family was discovered from the platyfish *Xiphophorus maculatus* and *Xiphophorus helleri* and was designated neurotrophin-6 (NT-6) (Götz *et al.*, 1994). Alignment of amino acid sequence with other neurotrophins suggested that NT-6 was structurally more related to NGF. However, NT-6 contained an additional feature not shared by all other known neurotrophins: an insertion of 22 amino acids between the second and third cysteine residues of the mature molecule which contained the heparin-binding domain. Although NT-6 was able to support the survival of chick sympathetic and dorsal root ganglion neurons, its potency was much lower than that of mouse NGF, probably reflecting low conservation of the molecule during evolution.

We report here the cloning of a novel neurotrophin from the fish, carp, identified using polymerase chain reaction (PCR) with primers designed from two highly conserved regions of *Xiphophorus* NT-6. This novel neurotrophin, named NT-7, satisfied the structural criteria that defined the neurotrophin family. Moreover, it contained an insertion of 15 amino acids at the position corresponding to that of *Xiphophorus* NT-6.

RESULTS

Molecular Cloning of Carp NT-7 and NGF

A DNA fragment of 340 bp was amplified from carp genomic DNA using primers corresponding to two conserved regions of NT-6 (Fig. 1A). Subsequent cloning

A

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1   CCAGGCCCGCGAGTCCGGCGGAAGGCCAACGACTTCTTGCATCGCGGCGAGTACTCTGTGTGTGACAGCGAAGAGCACTGGGTTGGCAAC
1   P  G  P  R  V  R  R  K  A  N  D  F  L  H  R  G  E  Y  S  V  C  D  S  E  E  H  W  V  G  N
91  CTGACCCAAGCCACAGACTTACGGGGCAATGAAGTCACGGTGCTGCCACATGTTCGCATCAACAACGTGGTGAAGAAGCAGATGTTCTAC
31  L  T  Q  A  T  D  L  R  G  N  E  V  T  V  L  P  H  V  R  I  N  N  V  V  K  K  Q  M  F  Y
181 GAGACCACGTGCCGTGTGTCGAAGCCCATCGGGGCCCCCAAGCCGGGTCAAGGAGTCAGCGGCGTTAAAGCAGGAACCTCTAGCTGTCGT
61  E  T  T  C  R  V  S  K  P  I  G  A  P  K  P  G  Q  G  V  S  G  V  K  A  G  T  S  S  C  R
271 GGGATCGACAACGAGCACTGGAACTCTTATTGCACCAACGTGCACACCTTTGTGCGGGCGTTAACGTCCTACAAAAACCAGATTGCCTGG
91  G  I  D  N  E  H  W  N  S  Y  C  T  N  V  H  T  F  V  R  A  L  T  S  Y  K  N  Q  I  A  W
361 AGGTTCATCCGAATCAACGCCGCTTGCGTGTGCGTCCTCAGCCGCAACTCATGGAGGCATTGACTGACATATTGTTTCAGCCAATCCACT
121 R  F  I  R  I  N  A  A  C  V  C  V  L  S  R  N  S  W  R  H  *
451 GCAGCCTCCTGTCGTAAGCCCCTCCCACCCATCAATAATAACAACAGCCGCACTGCCAACGTTGGTGAT
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B

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1   TTCTACGAAACGACGTGCAGCAGCGGGCGGACTGGAGGATCCGGGTGTTTGGGAATCGATGCACGCCATTGGAACTCGTACTGCACCAAT
1   F  Y  E  T  T  C  S  S  G  R  T  G  G  S  G  C  L  G  I  D  A  R  H  W  N  S  Y  C  T  N
91  TCACACACGTTTGTGCGAGCGCTGACTTCATTCAAGAACCTGGTGGCATGGAGACTCATAAGAATCAATGTAGCCTGCGTCTGCGTC
31  S  H  T  F  V  R  A  L  T  S  F  K  N  L  V  A  W  R  L  I  R  I  N  V  A  C  V  C  V
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FIG. 1. Nucleotide and deduced amino acid sequence of carp NGF and NT-7. (A) The basic motif RVRR (double underlined) was the putative proteolytic cleavage site, followed by an open reading frame of 133 amino acids that constituted the mature protein (starting with the lysine residue as indicated by the arrowhead). Exact oligonucleotides at two conserved regions of *Xiphophorus* NT-6 (corresponded to nucleotide 51 to 68 and 374 to 390 of NT-7) were used to perform the initial PCR from carp genomic DNA that generated the partial sequence of NT-7. The conserved cysteine residues in the mature region, as well as the conserved sequence nearby, were underlined. There is a characteristic insertion of 15 amino acids between the second and third cysteine residues (bolded). Note the entire pre–pro region that contained the putative ATG start codon followed by the signal sequence was not obtained. Sequence analysis of another clone revealed the same sequence except difference in nucleotides 141, 195, 208, and 283, with changes at Ile^{70} and Glu^{95} to Val and Lys, respectively. (B) Partial nucleotide sequence and the deduced amino acid sequence of carp NGF. A 177-bp fragment corresponded to the partial sequence between the second and the fifth conserved cysteine residues of carp NGF. The underlined sequence represented positions of the degenerate primers.

and DNA sequence analysis suggested the fragment might represent partial sequence of a novel neurotrophin. Northern blot analysis revealed its relatively strong expression in skin (see below). Therefore, in order to obtain the full-length sequence, 5′- and 3′-RACE (rapid amplification of cDNA ends) was performed using carp skin cDNA as template. The full-length clone which contained the putative translation start codon ATG and the signal peptide was not generated by the 5′-RACE. However, DNA sequence of the entire mature region could be obtained from the resulting clones. Subsequently, PCR using primers designed from sequence at the pre–pro and the 3′-untranslated region was performed to amplify the entire mature region.

DNA sequence analysis revealed that the pre–pro region was terminated at the two basic amino acids, arginine, followed by a mature region of 133 amino acids (Fig. 1A). Indeed, the R-X-K/R-R sequence was conserved in all neurotrophins and represented the proteolytic cleavage site, at which the mature protein was cleaved from its larger precursor (Hosaka *et al.*, 1991). The presence of all 6 conserved cysteine residues together with the nearby conserved regions in the mature protein suggested that it represented a novel neurotrophin molecule. Amino acid alignment of this novel neurotrophin, designated NT-7, with that of *Xiphophorus* NGF and NT-6 revealed 66% identity (Fig. 2A). Moreover, alignment with carp BDNF, chick NT-3, and *Xenopus* NT-4 suggested even more distant relationships (Fig. 2A). However, NT-7 lacked some amino acid residues which were conserved in all NGF molecules identified so far (Fig. 2B).

Because of its structural similarity to NGF, it is

A

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Carp NT-7          KA-NDFLHRGE YSVCDSEEHW VGN--LTQAT DLRGNEVTVL PHVRINNVVK KQMFYETTCR VSKPIGAPKP GQGVSGVK--
Xipma NT-6         ..VSHTM.... ......INT. .N---K.R.. .MS....... S..TV..K.. ..L....... SPTHRSSGIV IGGRSGGRGG
Carp NGF                                                                          S SGR------- ----------
Xipma NGF            QPQ...V ....E.VSV. ...--K.K.. .IS.K..... .Y.N....K. ..Y.F....H SPP------- ----------
Carp BDNF          HSDPAR... L.....ISQ. .TALDKKT.I .MS.QT.... EK.PVT.GQL ..Y....K.N PLG------- ----------
Chick NT-3         YAEHKS.... .......SL. .TD--KSS.I .I..HQ.... GEIKTG.SPV ..Y....R.K EAK------- ----------
Xen NT-4       ASGSDSVSLSR... L.....VNV. .TD--KRT.V .D..KI...M SEIQTLTGPL ..Y.F..K.N P.G------- ----------

Carp NT-7          -----AGTSS CRGIDNEHWN SYCTNVHTFV RALT-SYKNQ -IAWRFIRIN AACVCV-LS- RNSWRH
Xipma NT-6         KQGSKT.N.G .....SRY.. .H...TDIY. S...-VF.E. -T........ ......-..- ....SRRPGH
Carp NGF           -----T.G.G .L...AR... .....S.... ....-.F..L -V...L.... V
Xipma NGF          -----S.G.R .L...AR... .H...S.... ....-.SE.. -V...L.... V.....-..- .K..Q.
Carp BDNF          -----YTKEG .....KR.Y. .Q.RTTQSY. .....-MDSKR K.G......D TS...T-.TI KRGR
Chick NT-3         -----PVKNG .....DK... .Q.KTSQ.Y. ....-.EN.K LVG..W...D TS...A-..- .KIG.T
Xen NT-4           -----STTRG ...V.KKQ.I .E.KAKQSY. ....IDANKL -VG..W...D T....TL..- .TGRT
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B

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Carp NT-7          KANDFLHRGE YSVCDSEEHW VGNLTQATDL RGNEVTVLPH VRINNVVKKQ MFYETTCRVS KPIGAPKPGQ
Carp NGF                                                                      SSG R---------
Xipma NGF            QPQ...V ....E.VSV. ...K.K...I S.K......Y .N....K... Y.F....HSP P---------
Salmon NGF                                                                    ...GA R---------
Cobra NGF          EDHPVHNL.. H.....VSA. .-TK.T...I K..T...MEN .NLD.K.Y.. Y.F..K.KNP N---------
Xen NGF            TVHPV..K.. ......VSM. ..EK.K...I K.K.....GE .N...S.F.. Y.F..K..DP K---------
Chick NGF          TAHPVL.... F.....VSM. ..DK.T...I K.K.....GE .N...N.F.. Y.F..K..DP R---------
Mouse NGF          SSTHPVF.M.. F.....VSV. ..DK.T...I K.K.....AE .N...S.FR. Y.F..K..A. N---------
Bovine NGF         SSSHPVF.... F.....ISV. ..DK.T...I K.K..M..GE .N...S.F.. Y.F..K..DP N---------
Human NGF          SSSHPIF.... F.....VSV. ..DK.T...I K.K..M..GE .N...S.F.. Y.F..K..DP N---------

Carp NT-7          GVSGVKAGTS SCRGIDNEHW NSYCTNVHTF VRALTSYKNQ IAWRFIRINA ACVCVLSRNS WRH
Carp NGF           ------T.G. G.L...AR.. ......S... ......F..L V...L....V
Xipma NGF          ------S.G. R.L...AR.. ..H...S... ......SE.. V...L....V ........K. .Q.
Salmon NGF         ------..S. G.L...GR.. ......S... ......F.DL V.
Cobra NGF          ------PEP. G.....SS.. .....ETD.. IK...MEG.. AS......ET .....ITKKK GN
Xen NGF            ------PVS. G.....AK.. .....TT... .K...MEGK. A.......DT ........KG RT
Chick NGF          ------PVS. G.....AK.. .....TT... .K...MEGK. A.......DT ........KS G.P
Mouse NGF          ------PVE. G.....SK.. .....TT... .K...TDEK. A.......DT ........KA T.
Bovine NGF         ------PVD. G.....AK.. .....TT... .K...MDGK. A.......DT ........KT GQRA
Human NGF          ------PVD. G.....SK.. .....TT... .K...MDGK. A.......DT ........KA V.RA
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FIG. 2. Alignment of carp NGF and NT-7 with other neurotrophins. (A) Alignment of NGF and NT-7 with other known neurotrophins from lower vertebrates (Xipma represented *Xiphophorus maculatus*; Xen represented *Xenopus laevis*). Amino acids identical to that of NT-7 were represented by dots, while gaps were indicated by dash. Conserved regions among all the neurotrophins were underlined. The 15-amino-acid insertion (double underlined) was characteristic to this novel neurotrophin. The mature region of NT-7 shared 66% amino acid identity to Xipma NGF and NT-6 (without taking into account the insertion). (B) Alignment of carp NGF and NT-7 with NGF from various animals. Only partial sequence of salmon NGF had been reported. The amino acid residues that were conserved in all known NGF but different in NT-7 were bolded.

possible that NT-7 merely represents the carp homologue of NGF. However, Southern blot analysis of carp genomic DNA revealed that different DNA fragments hybridized with *Xiphophorus* NGF and carp NT-7 (data not shown), suggesting that NGF and NT-7 represented different genes. To clone the carp NGF homologue, PCR was performed using several pairs of degenerate primers that corresponded to conserved regions near the cysteine residues. As a result, a 177-bp fragment was amplified from carp genomic DNA by one of the pairs of primers (Fig. 1B). Analysis of the deduced amino acid sequence indicated its close resemblance to NGF; it shared 81, 67, 60, 50, 40, and 35% amino acid identity to the corresponding region of *Xiphophorus* NGF, carp NT-7, *Xiphophorus* NT-6, chick NT-3, carp BDNF, and *Xenopus* NT-4, respectively (Figs. 2A and 2B). In addition, it lacked those amino acid residues that were highly conserved in either BDNF, NT-3, or NT-4 of different species (Hallböök *et al*., 1991). These findings strongly suggested that this clone represented the partial sequence of carp NGF.

Southern Blot Analysis of Xiphophorus *Genomic DNA*

To examine whether NGF, NT-6, and NT-7 represent three different genes that coexist in one genome, Southern blot analysis of *Xiphophorus* genomic DNA, digested by *Hin*dIII, was performed and hybridized with *Xiphophorus* NGF, NT-6, or carp NT-7 cDNAs under low stringency conditions. It was found that different patterns were obtained by hybridization with the three neurotrophin cDNAs. The DNA fragments that hybridized with NGF were ≈8 and ≈3 kb, while a single fragment of ≈8 and ≈2 kb hybridized with NT-6 and NT-7, respectively (Fig. 3). This suggested that NGF, NT-6, and NT-7 did represent three different genes.

Spatial Expression of NT-7 in Adult Carp

The spatial expression of NT-7 in adult carp was studied by Northern blot analysis. A single transcript of about 1.1 kb was detected in skin and heart, though weak expression was also found in brain and intestine (Fig. 4A). This was in contrast with that of NT-6, where expression in adult fish was predominantly found in brain, gill, liver, and eye, but not skin (Götz *et al*., 1994).

Furthermore, the expression of NGF was studied in adult *Xiphophorus* in order to compare its spatial expression with that of NT-7. Northern blot analysis revealed dominant expression of a single transcript of ≈3.6 kb in eye and gill, though weak expression could be detected in skin (Fig. 4B).

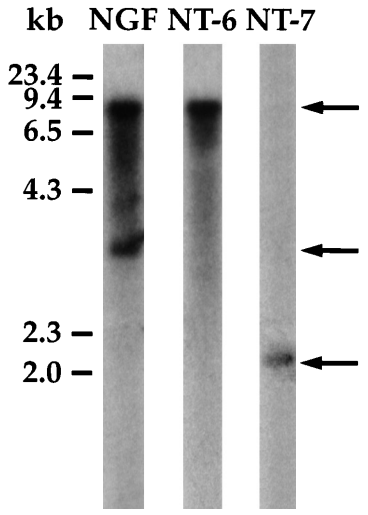

FIG. 3. Southern blot analysis of *Hin*dIII-cut *Xiphophorus* genomic DNA. The digested DNA was hybridized with cDNA of *Xiphophorus* NGF, NT-6, or carp NT-7 under low stringency. Numbers on the left indicated the sizes of the *Hin*dIII-digested lambda marker DNA (in kilobases). Distinct bands of ≈8 and ≈3, ≈8, and ≈2 kb hybridized with *Xiphophorus* NGF, NT-6, and carp NT-7, respectively.

Presence of the 15-Amino-Acid Insertion in the NT-7 Transcript as Determined by RT–PCR

One of the interesting features of NT-7 was the insertion of 15 amino acids between the second and third cysteine residues, a feature not found in any other known neurotrophins (including *Xiphophorus* NT-6 which contained an insertion of 22 amino acids at the corresponding position). The insertion contained only 4 glycine and 2 basic amino acid residues compared to the 8 glycine and 6 basic amino acid residues in *Xiphophorus* NT-6 that corresponded to the heparin-binding domain. In order to rule out the possibility of the insertion being an intron, RT–PCR was performed using a pair of primers flanking the insertion. All the tissues that were shown to express NT-7 in the Northern blot produced a single band which corresponded to the size having the insertion. Moreover, the band was absent when RT was performed using the same RNA samples but without reverse transcriptase (Fig. 5). Thus, the resulting product which contained the insertion was not originated from contaminating genomic DNA, and there was no detectable alternative form of the NT-7 transcript that lacked the insertion.

Construction of NT-7 and Truncated NT-7 That Lacked the Insertion

Although the entire pre–pro region of NT-7 was not obtained, its biological activity could still be determined

upon fusion of its mature region with the pre–pro portion of *Xiphophorus* NGF in order to express the protein. Moreover, it was reported that NT-6 might bind to cell surface through the heparin-binding domain, which would hinder its release into the conditioned medium after transfection. With the assumption that the 22 amino acids that corresponded to the heparin-binding domain are not essential in its biological activity, a truncated form of NT-6, named NT-6(D22), was constructed in which the 22 amino acid insertion was deleted in order to assay its neurotrophic activity in conditioned medium. Since NT-7 was expressed using the *Xiphophorus* NGF pre–pro region, two additional constructs of NT-6 and NT-6(D22), with pre–pro exchange, were made. Furthermore, the 15 amino acid insertion in NT-7 might lead to its binding to extracellular matrix, just like NT-6. Accordingly, a truncated form of NT-7 that lacked the 15-amino-acid insertion, NT-7(D15), was also constructed.

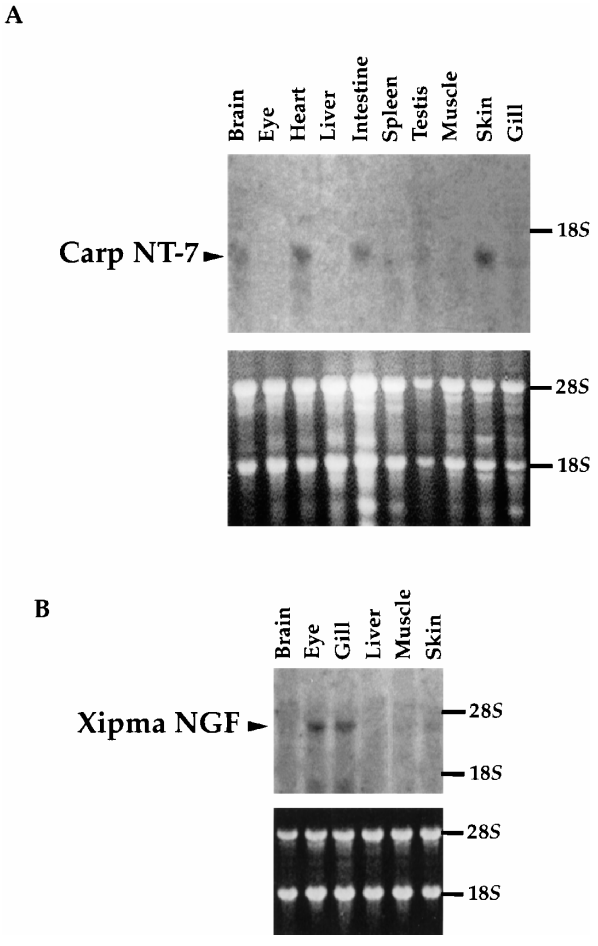

FIG. 4. Northern blot analysis of the spatial expression of NT-7 and NGF in adult fish. (A) Spatial expression of NT-7 in adult carp was examined. A single transcript of ≈1.1 kb was detected in skin and heart. Weak expression was also found in brain and intestine. (B) Spatial expression of NGF in *Xiphophorus*. A transcript of ≈3.6 kb was detected predominantly in eye and gill, while expression in skin was much weaker. In both A and B, the corresponding ethidium bromide-stained gel indicated similar amount of RNA loaded in each lane.

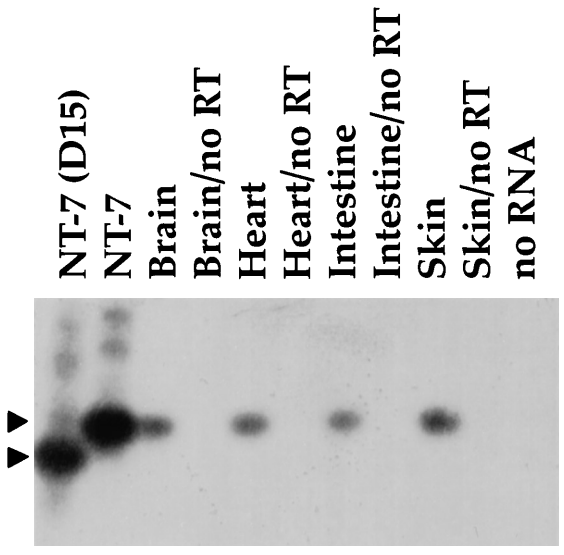

FIG. 5. RT–PCR showing the presence of the 15-amino-acid insertion in the transcript of NT-7. A fragment of 304 bp (indicated by upper arrowhead), which corresponded to the presence of the insertion, was produced after amplification from brain, heart, intestine, and skin cDNA by a pair of primers flanking the insertion (nucleotide 69 to 85 and 358 to 373 in Fig. 1). The RNA was replaced by DEPC-H_2O in the no-RNA control, while the same concentration of RNA but without reverse transcriptase was used in the no-RT control. NT-7 and NT-7(D15) represented PCR products amplified by the same primers from the expression plasmids NT-7 and NT-7(D15) which lacked the 15-amino-acid insertion (indicated by lower arrowhead), respectively. These served as the size standards to demonstrate that the insertion was present in the transcripts from the various tissues.

Biological Activities of NT-7

All the expression constructs were transiently transfected into COS-5 cells and conditioned medium was collected after 3 days. Neurite outgrowth assay of chick embryonic dorsal root ganglia (DRG) was employed to test the biological activities of the various neurotrophins. Addition of either *Xiphophorus* NGF or NT-7 resulted in robust neurite outgrowth from E8 DRG (Fig. 6). Moreover, NT-7(D15) also showed comparable activity, suggesting that NT-7 was still active without the 15-amino-acid insertion. In contrast, the effect of either NT-6 or NT-6(D22) was similar to that obtained from conditioned medium of mock-transfected cells. Similar

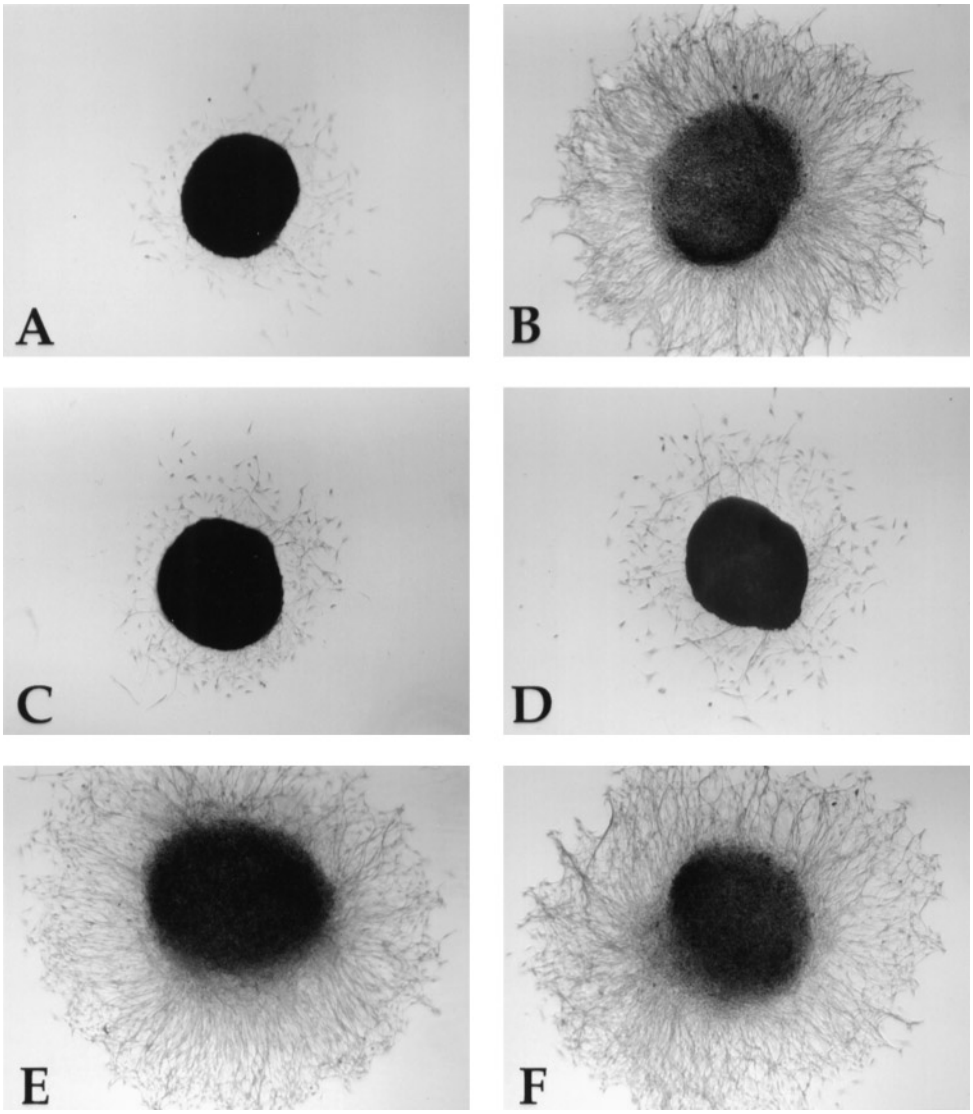

FIG. 6. Biological activities of the various fish neurotrophins. Photomicrographs showing E8 chick DRG after 1 day treatment with COS cell supernatant from mock-transfected cells (A), or from cells transfected with *Xiphophorus* NGF (B), NT-6 (C), NT-6(D22) (D), NT-7 (E), and NT-7(D15) (F). At least five DRGs were placed in each 35-mm dish coated with 1 μg/ml poly-D-lysine and treated with 1:5 diluted COS cell supernatant. The DRG were fixed and stained by anti-neurofilament 160-kDa antibody. Extensive neurite outgrowth was observed from DRG treated with *Xiphophorus* NGF, NT-7, or NT-7(D15) but not NT-6 and NT-6(D22). After pre–pro exchange with *Xiphophorus* NGF, NT-6 and NT-6(D22) were still unable to induce neurite outgrowth of DRG explant (data not shown). Magnification: ×60.

results were observed for the two NT-6 constructs with pre–pro exchange (data not shown). The neurotrophic activity of NT-7 was further investigated by the survival assay of dissociated DRG neurons. Consistent with the results of the neurite outgrowth assay, both NT-7 and NT-7(D15) could support the survival of E8 chick DRG neurons (Fig. 7). On the other hand, NT-6 and NT-6(D22) failed to promote significant neuronal survival, irrespective of the pre–pro region. Thus, the neurotrophic activity of NT-7 was demonstrated by its ability to stimulate neurite outgrowth and survival of DRG neurons, in a manner similar to that induced by *Xiphophorus* NGF.

Phosphorylation of Trk Receptors by NT-7

To elucidate its mechanism of action, the interaction between NT-7 and the various Trk receptors was studied by phosphorylation of different Trk receptors ectopically expressed in fibroblasts. Because of its structural

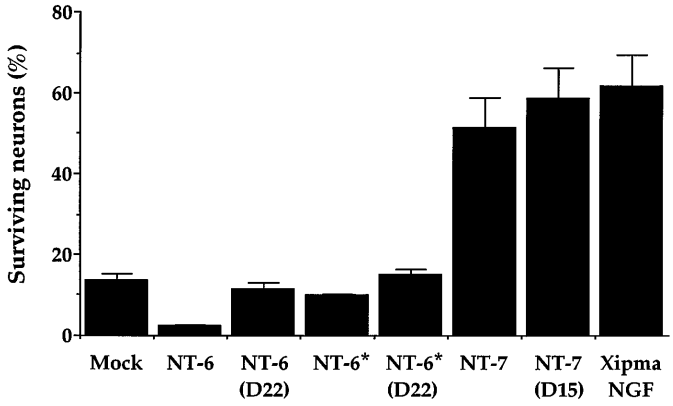

FIG. 7. Survival of dissociated DRG neurons in response to the neurotrophins. Dissociated DRG neurons from E8 chick were plated onto 35-mm poly-D-lysine-coated plates, and the number of surviving neurons were counted after 1-day treatment with the COS cell supernatant (diluted 6:5 by medium). *Xiphophorus* NGF, NT-7, and NT-7(D15) were able to support survival of the neurons (50–60%) while less than 20% of the neurons survived in response to NT-6, NT-6*, NT-6(D22), or NT-6*(D22), which was similar to that of mock-transfected cells. NT-6* represented NT-6 with pre–pro region of NGF. The error bars represented SEM, $n = 6$.

similarity to NGF, it would be expected that NT-7 showed receptor specificity to TrkA. Indeed, compared with the conditioned medium of mock-transfected COS cells, NT-7, NT-7(D15), and NT-6(D22) could weakly phosphorylate TrkA but not TrkB or TrkC (Fig. 8). It should be noted that despite the relatively weak level of TrkA phosphorylation, it was consistently observed in triplicate experiments. The extent of TrkA phosphorylation elicited by NT-7, NT-7(D15), and NT-6(D22) was considerably lower than that obtained by *Xiphophorus* NGF. NT-6(D22) failed to promote neurite outgrowth and survival of DRG, yet its induction of TrkA phosphorylation was similar to NT-7 and NT-7(D15). On the other hand, with the presence of the 22-amino-acid insertion, NT-6 was unable to stimulate any detectable level of TrkA phosphorylation, probably because of its binding to the cell surface or extracellular matrix, which subsequently hindered its release to the conditioned medium. Similarly, NT-6(D22) with NGF pre–pro region could induce weak TrkA phosphorylation (data not shown).

Construction of Fc-Tagged NGF and NT-6 and Assay for Neurite Outgrowth Activity

Since NT-6 had been demonstrated to promote the survival of chick DRG neurons (Götz *et al*., 1994), the negative activity of NT-6 or NT-6(D22) in our study might be explained by lower expression and / or potency of the neurotrophin compared with NGF. To address this question, constructs of *Xiphophorus* NGF, NT-6, and NT-6(D22) were tagged with the Fc region of human IgG. The relative amount of each neurotrophin in the conditioned medium was then determined by ELISA (see Experimental Methods), and their ability to stimulate neurite outgrowth of chick DRG was assayed after equalizing the amount of each neurotrophin.

It was found that only NGF–Fc could stimulate the neurite outgrowth of DRG, while the activity of NT-6(D22)–Fc as well as NT-6–Fc (data not shown) was similar to that of conditioned medium of mock-transfected cells (Fig. 9). Therefore, the different response of DRG to the two neurotrophins in our study was likely due to the lower potency of NT-6 in promoting neurite outgrowth. Indeed, the EC50 of purified NT-6 in supporting the survival of chick DRG was quite high (about 100 ng / ml; Götz *et al*., 1994). It was therefore possible that the expression level of NT-6 and NT-6(D22) in our study was not high enough to reveal its activity on DRG neurons.

DISCUSSION

NT-6 was originally cloned from the aquarium fish *Xiphophorus maculatus* (Götz *et al*., 1994), but so far no homologue in any other vertebrate was found (Lai and Ip, unpublished data). With the assumption that NT-6 was highly homologous among different types of fish, particularly at the conserved regions around the cysteine residues, PCR was performed using a pair of primers at two conserved regions in order to clone the carp NT-6 homologue. It was therefore surprising that the resulting fragment, designated NT-7, only shared 66% amino acid identity to *Xiphophorus* NT-6. Apart

from the relatively low percentage of homology, there was also considerable differences between the primary structure of the two neurotrophins. For example, the lack of an amino acid between Asn[23] and Lys[24], which was characteristic to NT-6 but not any other known

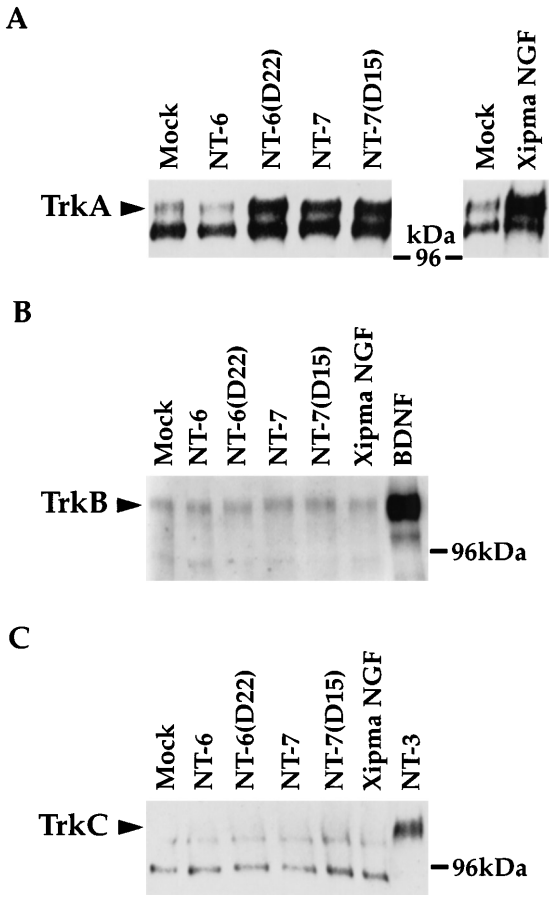

FIG. 8. Phosphorylation of chick Trk receptors by the neurotrophins. (A) Chick TrkA was transiently transfected into 293 fibroblasts and assayed for phosphorylation by the COS cell supernatant (undiluted) that contained the various fish neurotrophins. Similar observation was obtained using 6:5 diluted COS cell supernatant. The faster migrating band (about 96 kDa) represented constitutively phosphorylated precursor (Ibćñez *et al*., 1993), and its signal could serve to equalize the amount of sample on each lane. All the fish neurotrophins examined, except NT-6, could weakly phosphorylate TrkA, but the extent of phosphorylation was considerably lower than that of *Xiphophorus* NGF. Similarly, NT-6(D22), but not NT-6, that contained NGF pre–pro region could induce weak TrkA phosphorylation (data not shown). (B) Chick fibroblasts that were stably transfected with chick TrkB were used to test the ability of the neurotrophins to phosphorylate TrkB. Only BDNF (50 ng/ml), which served as the positive control, could phosphorylate the receptor. (C) Phosphorylation of TrkC by the neurotrophins was tested by chick fibroblasts stably transfected with chick TrkC. All the fish neurotrophins were unable to phosphorylate the receptor, except NT-3 (50 ng/ml) which acted as the positive control (since only one-third of the positive control was loaded, the lower two bands that were present in the other samples were not visible).

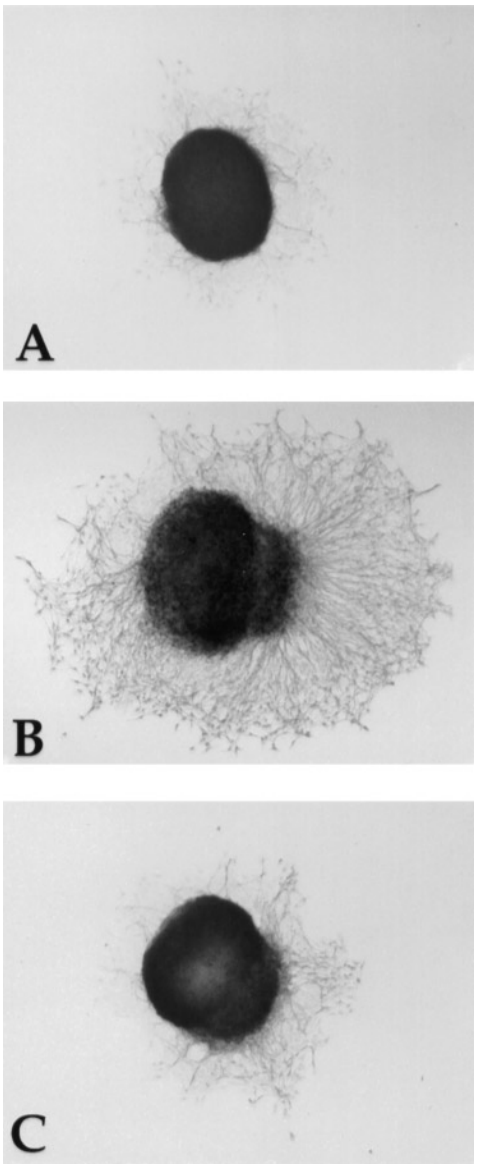

FIG. 9. Neurite outgrowth of DRG in response to Fc-tagged neurotrophins. COS cell supernatant that contained equal amounts of the Fc-tagged NGF (B) or NT-6(D22) (C), as determined by ELISA, was added to E8 chick DRG. Only NGF–Fc promoted the neurite outgrowth, while the activity of NT-6(D22)–Fc was similar to mock-transfected COS cell supernatant (A). The activity of NT-6–Fc was also similar to the mock (data not shown). Magnification: ×60.

neurotrophins, was not found in NT-7 (Fig. 2A). Moreover, the insertion present in NT-7 was considerably different from that of NT-6 in terms of the length and number of basic amino acid residues. Southern blot analysis demonstrated that NGF, NT-6, and NT-7 represented three different genes in the *Xiphophorus* genome. The partial sequence of carp NGF cloned in our study

indicated that NGF and NT-7 were indeed two different genes. Since the homology between NGF and NT-7 is very similar to that between NT-7 and NT-6 (about 66% in both cases), it supports our claim that NT-7 represents a different gene from NT-6, rather than the carp NT-6 homologue. In addition, the spatial expression of NT-7 in adult tissues was quite different from that of *Xiphophorus* NGF and NT-6. In particular, high level of expression of NT-6 was reported in adult brain, eye, and gill, while NT-7 expression in the brain was low, and was even undetectable in eye and gill. Taken together, despite the relatively high evolutionary rate of neurotrophin in lower vertebrates (Götz *et al*., 1992), NT-7 represents a novel member of the neurotrophin family.

In the present study, we have demonstrated the ability of NT-7 to stimulate neurite outgrowth and survival of E8 chick DRG. Unlike NT-7 and *Xiphophorus* NGF, NT-6 was unable to induce positive response from DRG in either assay. Although it has previously been suggested that the pre–pro region can affect the processing of mature neurotrophin (Ip *et al*., 1992), the pre–pro exchange with that of *Xiphophorus* NGF could not enable NT-6 to induce neurite outgrowth from DRG. It was possible that NT-6 secretion into the conditioned medium was hindered by its binding to cell surface or extracellular matrix, as suggested by Götz *et al*. (1994). However, NT-6(D22), which was expected to be released to the medium because of the absence of the heparin-binding domain, also could not activate the DRG in the neurite outgrowth and survival assay. Our results using Fc-tagged NT-6(D22) suggested that NT-6 was less potent than NGF in stimulating neurite outgrowth of chick DRG. This finding is indeed consistent with the results obtained in two separate studies (Götz *et al*., 1992, 1994), which suggested that NT-6 was much less potent than *Xiphophorus* NGF in promoting the survival of DRG: the concentration of *Xiphophorus* NGF causing half-maximal activity was 6 compared to 100 ng / ml of NT-6. It was possible that in our experiments, the concentration of the neurotrophin in COS cell supernatant was below the half-maximal value of NT-6 but not NGF, thus allowing for detectable response to NGF but not NT-6 in the DRG. Nonetheless, NT-6(D22) did exhibit the ability to induce weak TrkA phosphorylation.

Neurotrophins mediate their actions on neurons via phosphorylation of the Trk receptors. In our studies, the receptor specificity of NT-7 was determined by phosphorylation of chick Trks ectopically expressed in fibroblasts. As expected from the structural similarity to NGF, NT-7, NT-7(D15), and NT-6(D22) specifically phosphorylated TrkA. However, the phosphorylation was considerably weaker than that induced by *Xiphophorus* NGF. The weaker phosphorylation of TrkA by NT-6(D22) or NGF / NT-6(D22) was consistent with our hypothesis that NT-6 was less potent than *Xiphophorus* NGF in activating DRG neurons. However, NT-7 and NT-7(D15), despite their ability to activate DRG in both neurite outgrowth and survival assay, could only phosphorylate TrkA weakly. Indeed, the extent of TrkA phosphorylation was similar to that induced by NT-6(D22), which did not show any detectable activity on DRG in our studies. Both NT-7 and NT-7(D15) could not phosphorylate TrkB and TrkC, suggesting that the action on DRG was not mediated by these two Trk receptors. It remains possible that NT-7, but not NT-6, can activate a unique signaling pathway that mediates the response of DRG despite the modest activation of TrkA.

One important feature of NT-7 is the insertion of 15 amino acids at the position corresponding to the insertion of *Xiphophorus* NT-6. The insertion lacks the GT-AG sequence that is conserved in the intron / exon boundary. This, together with the result of RT–PCR, suggested that the insertion did not represent an intron. Since the activity of NT-7(D15) was as potent as NT-7 in both the DRG assay and the phosphorylation assay, the 15-amino-acid insertion was not indispensable in its biological activity. Its presence in the NT-7 molecule might be accessory to its neurotrophic function, such as binding to extracellular matrix which helped to localize the factor in the target area. If that is the case, it will be interesting to examine whether NT-7, like NT-6, binds to heparan sulfate or other types of molecules present in the extracellular matrix.

The cloning of this novel neurotrophin from carp shed new light on the diversity of neurotrophins in lower vertebrates. Considerable differences among NT-7, NGF, and NT-6 were observed in terms of primary structure and spatial expression. This, together with the Southern analysis of *Xiphophorus* genomic DNA and the low homology between NT-6 and NT-7 which is comparable with that of NGF, provide evidence that NT-7 is a novel neurotrophin.

EXPERIMENTAL METHODS

Molecular Cloning of Xiphophorus *NGF, NT-6, Carp NGF, and NT-7*

In an attempt to clone the carp NT-6, two exact oligonucleotides representing the amino acid sequence

YSVCDS (GTACTCTGTGTGTGACAG) and INAACV (CACACATGCAGCGTTGA), which corresponded to two conserved regions of NT-6, were designed (Fig. 1). Carp genomic DNA (0.6 µg) was used in the polymerase chain reaction (PCR) as template. One-tenth of the reaction was further amplified by the same set of primers in a second PCR. The fragment was then gel purified and the ends were blunted by Klenow (Amersham, UK) before being ligated to *Sma*I-cut pBluescript (Stratagene, CA). After transformation into XL-1 blue, the plasmid was purified and double-stranded DNA sequencing was performed (Sanger *et al*., 1977). The resulting fragment was designated as NT-7. Rapid amplification of cDNA ends (RACE) was employed in order to clone the full-length NT-7 from carp skin RNA. Methods were the same as stated in the protocol (Gibco, NY). The 5′- and 3′-RACE revealed the sequence of part of the pre-pro region and the 3′-untranslated region. To clone the entire mature region, two primers (AAATGATACGGGGAGCC and AAGGGGCGGAGTCTCAG) located at the pre–pro and 3′-untranslated regions, respectively, were used to amplify NT-7 from 1.3 µg of carp genomic DNA using Vent polymerase (New England Biolab, MA). The resulting fragment of 553 bp was subcloned into pBluescript by blunt-end ligation as mentioned above. The partial sequence of carp NGF was amplified by PCR from 0.3 µg carp genomic DNA by a pair of degenerate primers that corresponded to the conserved regions FYETTC and ACVCV. To clone the full-length *Xiphophorus* NGF and NT-6, two pairs of primers (CTTAGATCGTGTGCCCATG and GGGTGAGTCTTCAATGCTG for NGF; ATAACGTGGACGTGTGCCC and CAAGAGCGGTCCACACCTC for NT-6) were designed and PCR was performed using 1.8 µg *Xiphophorus* genomic DNA as template. The resulting products were subcloned into the expression vector pMT21. The cDNA sequence for carp NT-7 has been submitted to Genbank, under the Accession No. U94949.

Genomic Southern Analysis

Genomic DNA was prepared from 3 g of carp or *Xiphophorus* tissues as previously described (Ausubel *et al*., 1992). Genomic DNA (50 µg) was digested with 1500 units of *Hin*dIII for 6 h, after which it was further digested by 980 units of the enzyme for 16 h. Digested genomic DNA (16 µg) was loaded to each lane on 1% agarose gel. After being transferred to nylon membrane, it was hybridized at 50°C in sodium phosphate buffer containing 25% formamide.

RNA Extraction, Northern Analysis, and RT–PCR

Selected tissues were dissected from adult carp and immediately frozen in liquid nitrogen. Total RNA was extracted from homogenized tissues by lithium chloride precipitation or guanidium thiocyanate extraction as described previously (Ip *et al*., 1995). Total RNA (15 µg) was electrophoresed on a 1% agarose-formaldehyde gel, transferred onto a nylon membrane (MSI, MA), and cross-linked by UV irradiation (Stratalinker, Stratagene, CA). A 340-bp fragment corresponding to the partial sequence of NT-7 was used as the probe for Northern analysis. The DNA fragment was gel purified by Qiaex (Qiagen, Germany) and labeled by random priming (Megaprime labeling kit, Amersham, UK). RNA blots were hybridized at 65°C with radiolabeled probes in 0.5 M sodium phosphate buffer (pH 7), 1% bovine serum albumin, 7% SDS, 1 mM EDTA, and 20 µg / ml sonicated salmon sperm DNA. Filters were washed at 65°C with 2× SSC, 0.1% SDS, and exposed to X-ray film (XAR-5, Kodak) with intensifying screens (Model SQZ-, C.B.S. Scientific CO., Inc.) at −80°C.

For RT–PCR analysis, 2 µg of RNA from various carp tissues was used as template in the reverse transcription that utilized Superscript RT (Gibco, NY). The RNA was pretreated with 1 unit of RNase-free DNase (Promega, WI) for 30 min at 37°C. For the control, no RT was added and was substituted by DEPC-H_2O. One tenth of the reaction was amplified by two primers flanking the insertion (Fig. 1). The resulting product was analyzed in agarose gel and subjected to Southern blotting, using the same fragment used in the Northern blot analysis as probe.

Construction of Various Neurotrophins

A fusion protein that contained the pre-pro region of *Xiphophorus* NGF and mature region of NT-7 was constructed in order to test the activity of NT-7. The pre–pro region was amplified from the expression plasmid *Xiphophorus* NGF in pMT21 by sense primer located upstream of the *Xho*I cloning site of pMT21 and antisense primer that contained sequence at the end of the NGF pre–pro region plus 18 nucleotides from the beginning of the NT-7 mature region. The complementary sense primer and the antisense primer downstream of the *Not*I site of pBluescript were used to amplify the mature region of NT-7 (being subcloned into pBluescript). In a second PCR, the two fragments of DNA would anneal at the corresponding position because of the complementarity of the opposite ends, and the resulting NGF pre–pro / NT-7 chimera was amplified by the sense and antisense primers of pMT21 and pBlue-

script, respectively. The same approach was used to exchange the pre–pro region of NT-6.

To construct NT-7(D15) in which the 15-amino-acid insertion was deleted, a pair of complementary primers was designed which contained sequence immediately flanking the insertion. NT-7 (already fused with the NGF pre–pro region) encoded in pMT21 and pBluescript was used as template to produce the N- and C-terminal fragments, respectively. Because the two internal primers bypassed the insertion sequence, both the N- and C-terminal fragments did not contain the 15-amino-acid insertion. Using the two fragments as template in a second PCR (which involved sense and antisense primers of the two vectors) would generate NT-7(D15). A similar approach was employed to construct NT-6 lacking the 22-amino-acid insert. Fc-tagged NGF, NT-6, and NT-6(D22) were constructed by fusing the human Fc sequence at the C-termini of the neurotrophins as previously described (Yang *et al*., 1997). All the different neurotrophin DNAs were subcloned in the expression vector pMT21 and transformed into DH10B.

Expression of Various Neurotrophins and Assay for Biological Activities

All the expression constructs were transfected into COS-5 cells using the diethylaminoethanol (DEAE)-dextran-chloroquine method as previously described (Lai *et al*., 1996), except that 5 µg of plasmid was used in each transfection, and the cells were maintained in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS). Three days after transfection, the COS cell supernatant was collected and assayed for stimulation of neurite outgrowth from E8 chick DRG explants as described previously (Lindsay *et al*., 1985). The COS cell supernatant used in the assay was diluted 1:5 with DMEM + 10% FBS. The DRG were fixed and stained after 1 day treatment by anti-neurofilament 160-kDa antibody (Clone NN18, Sigma, MO) as previously described (Ip *et al*., 1991). Similarly, the neurite-outgrowth activities of the Fc-tagged neurotrophins were determined except the COS cell supernatant that contained NGF-Fc was diluted 1:2.5 and the amount of NT-6 and NT-6(D22) was correspondingly adjusted by mock-transfected COS cell supernatant. The relative amount of the Fc-tagged neurotrophins in the COS cell supernatant was determined by ELISA as previously described (Ausubel *et al*., 1992), using anti-human Fc antibody (Amersham, UK). Based on the absorbance of the COS cell supernatant, the amounts of protein added to the cultures were equalized (equivalent to OD of 0.290 ± 0.01 at 1:10 dilution, $n = 3$, $\pm$SEM)

To test whether the various neurotrophins could promote the survival of dissociated DRG neurons, the neurons were dissociated as previously described (Lindsay *et al*., 1985) and $3–4 \times 10^4$ cells (in DMEM + 10% FBS) were plated onto 35-mm plate coated with 100 ng / ml poly-D-lysine. COS cell supernatant (1.2 ml) was added to each plate in a final volume of 2.2 ml (i.e., the ratio of COS supernatant to medium was 6:5). The number of surviving neurons were counted after 20 h.

Phosphorylation Assay of Trk Receptors in Fibroblasts

To test TrkA phosphorylation induced by the neurotrophins, 293 cells were transiently transfected by chick TrkA (Beckstrom *et al*., 1996) encoded in the expression vector pCDM8, and 2×10^6 cells were plated onto a 100-mm plate. Twenty-four hours later, the cells were transfected with 9 µg DNA in 8 ml Opti-minimal essential medium (Opti-MEM, Gibco, NY) without penicillin / streptomycin but containing 40 µl lipofectamine (2 mg / ml, Gibco, NY). The cells were incubated with the liposome-DNA mix for 5 h at 37°C before replaced by DMEM + 10% FBS. The cells were refed at 20–24 h after the start of transfection. Three days later, $2–3 \times 10^6$ cells were plated onto each 100-mm plate for phosphorylation assay. After 24 h, the cells were starved in serum-free defined medium for 1 h and then 2.5 ml of the COS cell supernatant (undiluted) was added to the cells and they were incubated for 5 min at 37°C. The cells were lysed by RIPA buffer and assayed for Trk receptor phosphorylation as previously described (Lai *et al*., 1996). Similar experiment was performed using 6:5 diluted COS cell supernatant. This served to show that NT-6(D22) and NGF / NT-6(D22) could still induce the weak TrkA phosphorylation under the same dilution utilized in the DRG survival assay. To assay TrkB and TrkC phosphorylation, 2×10^6 chick fibroblasts stably transfected with full-length chick TrkB or TrkC were plated and the same procedure for TrkA phosphorylation was followed.

ACKNOWLEDGMENTS

We are grateful to Dr. Thomas H. Large for providing the stable lines of chick fibroblasts overexpressing full-length chick TrkB and TrkC, and to Dr. Frances Lefcort for the chick TrkA plasmid. We particularly thank Joanna S.L. Lau for her expert assistance in the survival assay of

dissociated DRG neurons. We also thank William M. W. Cheung and Amy K. Y. Fu for their helpful discussions as well as Wing-Sze Hue and Yin-King Lee for performing ELISA and DNA sequencing, respectively. This study is supported by the Research Grants Council of Hong Kong (HKUST 529/94M) and the Biotechnology Research Institute of HKUST.

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Received June 6, 1997
Accepted January 8, 1998